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14. ABSTRACT

We have designed and synthesized a novel compound (11ß) that efficiently triggers apoptosis in prostate cancer cells such as LNCaP. This bifunctional compound was designed to form DNA adducts that are camouflaged by the androgen receptor making them less readily repaired in AR+ prostate cancer cells. The aims of our studies are to investigate the mechanisms by which 11ß is able to trigger apoptosis in target cells. Methods have been developed that permit us to determine the fates of 11ß-DNA adducts in treated cells in culture as well as in tumors growing in animal models. Another objective is to identify the signaling events that lead from DNA adducts to activation of the apoptotic program. Finally we have obtained encouraging results from animal experiments that indicate that molecules such as 11ß may have clinical potential for the treatment of human tumors.

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INTRODUCTION

The objective of our research is to develop more effective therapeutics for the treatment of prostate cancers. One novel bifunctional compound (11β) that we have prepared rapidly induces apoptosis in several prostate cancer cell lines *in vitro*. The 11β compound (see Fig. 1) contains a chemically reactive nitrogen mustard linked to a steroid moiety that binds with high affinity to the androgen and progesterone receptor proteins. This compound was designed to create DNA adducts that form tight complexes with these steroid receptors that make the adducts difficult to repair in prostate cancer cells. Preliminary studies of 11β in cell culture indicated that its effects on prostate cancer cells were different from those of other alkylating agents used in chemotherapy. The apoptotic responses of prostate cancer cells suggested that the 11β compound might be a useful agent for chemotherapy. The Specific Aims of our research are to understand the fate of 11β-DNA adducts in treated cells and investigate the mechanisms that lead to apoptosis. We also proposed experiments to assess the antitumor potential of 11β in animal models of human prostate cancer.

BODY

Task 1: *Determine if the biochemical changes observed in prostate cancer cells in culture are responsible for the antitumor effects of 11β in xenograft tumor models*.

One biochemical change that we proposed as a marker of antitumor response was the DNA damage induced protein p21. Our investigation of p21 responses to 11β in the prostate cancer cell lines LNCaP, DU145 and PC3 have been completed. We expanded examination of the levels of p21 in the three cell lines up to 24 h after treatment with 11β. Our present results indicate that p21 responses are robust in LNCaP and PC3 cells, but this was not the case in the DU145 cell line. Therefore, the p21 marker is not appropriate an appropriate biomarker for all of our tumor models.

We examined the feasibility of using increased expression of the p21 protein as a marker of tumor response in xenografts. Immunohistology was use to detect increased expression of p21 in tissue sections after treatment with 11β. These techniques proved not to be sensitive enough to determine their responses in the heterogenous cell populations of cells present in xenograft tumors. As the p21 protein was the most intense response we have seen in vitro we decided not to continue the strategy of using protein expression changes as biomarkers *in vivo*. We focused our attention on Task 2 since the measurement of DNA adducts would provide a quantitative measure of the amounts of 11β that reached the tumor.

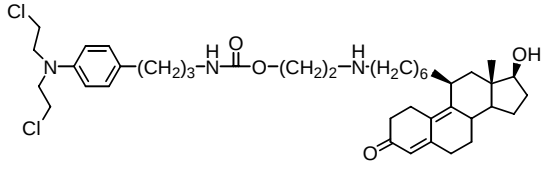

Figure 1. Structure of 11β

Task 2: *Determine the fate of 11β-DNA adducts in prostate cancer cells and in LNCaP xenograft tumors in animals.*

Previous progress reports during the current grant period described studies using AMS and LC/MS to detect and quantify the levels of adducts in prostate cancer cells in culture and in tumor tissues. Using a radiolabeled analog that incorporated one ^{14}C atom into the linker of our molecule we have used the technique of Accelerator Mass Spectrometry (AMS) to detect and quantify [^{14}C]-11β-DNA adducts in treated cells. This permitted us to define the dose-response relationship between the concentration of 11β in cell culture media and the level of total DNA adducts in cells (Fig.2). Since the cytotoxic effects of 11β in LNCaP cells are well defined we were able to obtain a relationship between DNA adduct levels and toxicity. (Fig 3). We then compared the DNA adduct levels in LNCaP xenograft tumors growing in mice after the animals were treated with [^{14}C]-11β at doses that produced an antitumor response. These experiments revealed that the adduct levels achieved in xenografts after a single dose of 11β (0.14 to 0.24 11β-DNA adducts/10^6 bases) were within the range of those that showed toxic effects in our cell culture based experiments. Based on these results we think that 11β-DNA adduct levels will provide a useful metric with which to correlate toxicity *in vitro* with antitumor responses *in vivo*.

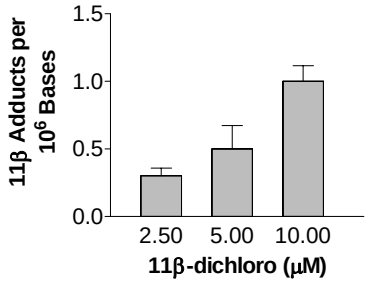

Figure 2. Formation of 11β-DNA adducts in LNCaP cells treated with [^{14}C]11β for 2 hr. (Hillier et al. 2006 Mol. Cancer Ther. 5; 977)

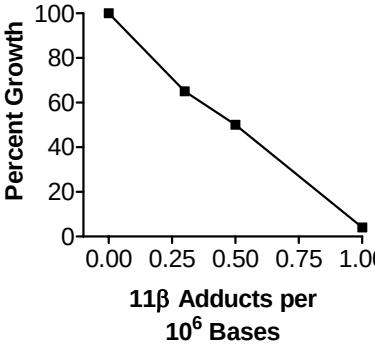

Figure 3. Relationship between the level of 11β DNA adducts and percent growth of LNCaP cells in culture.
(Hillier et al. 2006 Mol. Cancer Ther. 5; 977)

Despite the extraordinary sensitivity of AMS, a major drawback of this technology is the lack of structural information. The majority of adducts formed are monoadducts while the ability of 11β-dichloro monoadducts to subsequently form crosslinks is essential for the compound's potent cytotoxic effects. We demonstrated this by preparing an 11β-monochloro compound that was capable of only forming monoadducts. The 11β-monochloro analog that cannot form DNA crosslinks is much less toxic than the 11β-dichloro. This result suggests that optimization in tumor cells of crosslink formation by 11β-dichloro would lead to better tumor response and that monitoring the formation of DNA crosslinks and their fate in cells would best correlate with tumor response.

To complement our ability to measure total DNA adducts by AMS we developed a method based on liquid chromatography-electrospray ionization/tandem mass spectrometry that permits the analysis of 11β-DNA adducts. To ascertain the relative amounts of 11β-guanine and 11β-adenine monoadducts, as well as 11β-guanine cross-links *in vivo*, we have developed a quantitative HPLC-ESI^+-MS/MS method for measuring 11β-DNA adducts extracted from cell and tissues. The adducts are released

from DNA as bases via mild acid hydrolysis, purified by solid phase extraction and subjected to ESI^+-MS/MS analysis using an Agilent 6410 triple quadrupole electrospray ionization mass spectrometer. Doubly protonated forms of 11β adducts are selected for collision induced dissociation which results in the release of the protonated base. Selected reaction monitoring was preformed for the formation of guanine cations from 11β-guanine monoadducts (*m/z* 407.5 $[M+2H]^{+2}$ → 152.1 $[Gua + H]^+$) or bis-Gua-11β crosslinks (*m/z* 474.3 $[M+2H]^{+2}$ → 152.2 $[Gua + H]^+$) as well as for adenine cations from 11β-adenine monoadducts (*m/z* 399.5 $[M+2H]^{+2}$ → 136.1 $[Ade + H]^+$). The limit of detection of our current method is 10-20 fmol which is based on analysis of amounts of radiolabeled adducts formed *in vitro* by [^{14}C]-11β (Specific activity = 42 mCi/mmol). The ability of this method to detect and quantify 11β adducts in treated cells is shown in Figure 4. LNCaP cells (~ $2x10^6$) were treated for 6 h with 5 uM 11β. The levels of both monoadducts and crosslinks appear to be well within the lower limit of quantification with this method.

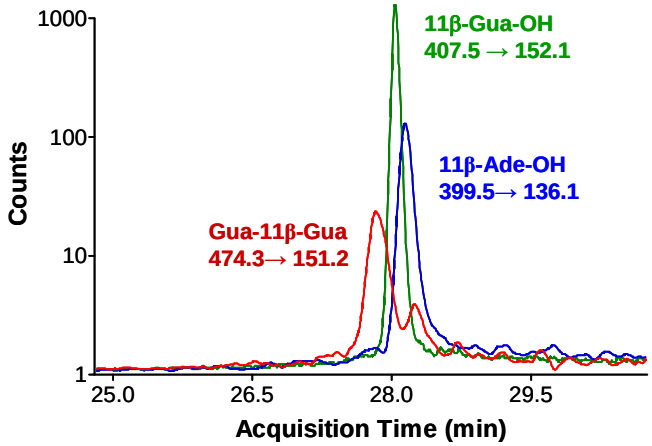

Figure 4. HPLC-ESI^+-MS/MS analysis of 11β-DNA adducts isolated from LNCaP cells treated with 5 uM 11β for 6 h.

We are now at the point of being able to test whether these techniques have the required sensitivity for quantitative analysis of monoadducts and crosslinks in tissues of treated animals. These 11β-DNA adducts will server as biomarkers that can be used to quantify the doses of compound experienced by both target and nontarget tissues under various dosing regimens. This should enable us to rapidly evaluate different formulations and dosing schedules to optimize dosage to the tumor tissue. We anticipate applying these methods to the xenograft models described in Task 4.

Task 3: *Formulation of 11β-dichloro in a liposomal vehicle and investigation of its PK and efficacy.* .

Our original plan was to investigate liposomal formulations of 11β. This Task was based on promising preliminary results in which 11β was incorporated into liposomes via membrane extrusion. The 11β molecules exhibited greater stability to hydrolysis in aqueous solution and had an extended half life in blood after i.v. administration. During the current grant period, changes in personnel both in our lab and those of our collaborator delayed our original plans to investigate this new approach to formulate the compound. Furthermore, discussions with several oncologists led us to recognize the advantages of oral administration of the 11β compound both in terms of speed in reaching the clinical trial stage and future clinical acceptance. Therefore, we decided to explore the bioavailability of 11β-dichloro in the mouse after oral dosing. As we described in our FY2008 Annual Report, the results of our experiments in mice indicated that following

administration via gavage, the bioavailability of 11β was very low with most to the compound remaining unabsorbed in the gut (Fig 5). Further experiments directly involving formulations were postponed until we developed a better understanding of the physical chemical properties (e.g., solubility) and metabolic fate of the compound.

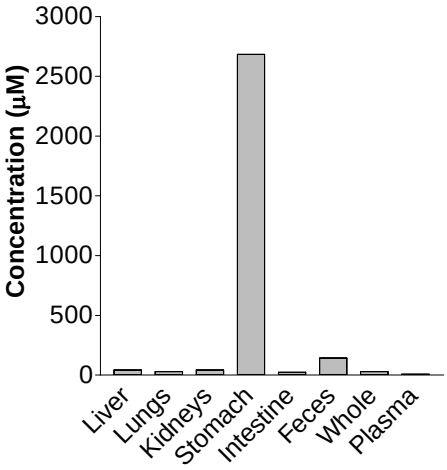

Figure 5. Concentrations of [^{14}C]-11β in tissues 6 h after administration of 130 mg/kg via gavage.

We recognized that solubility in aqueous solutions was an important issue affecting the formulation of our compound and its route of administration to animals. As a model system – and because our toxicity studies were carried our in cell culture – we examined the solubility of 11β in cell culture media and assessed the influence of media components on its solubility. Radiolabelled 11β was added to Minimal Essential Media (MEM) with and without 10% fetal calf serum (FCS) at 37^{o}C. The media was then centrifuged to remove any suspended or insoluble material and the amount of [^{14}C]-11β remaining in solution was determined by liquid scintillation counting. The results shown in Fig. 6 indicate that the compound has very low solubility in MEM in the absence of serum. Less than 10% of the added 11β remained in MEM after centrifugation – even at the lowest concentration tested. The presence of serum which contains high concentrations of proteins and lipids increased solubility with >90% of the compound remaining in the supernatant after centrifugation. These results make it likely that the compound is tightly bound to serum components following administration *in vivo*. This knowledge has been of importance in our understanding of delivery of the active compound to the tumor site. We observed a half-life for the hydrolysis of the *N,N*-bis-2-chloroethyl group of ~30 min in aqueous solutions which implied that the formation of DNA adducts in target tissues could be limited by rapid inactivation of the alkylating group. In the presence of 10% serum, however, there was a considerably slower rate of inactivation; the half life was ~ 6 h. Thus, the association of 11β with serum components is likely important for delivery of the active molecule to target tissues.

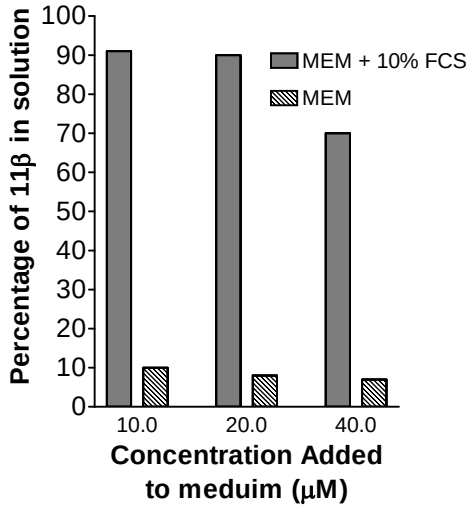

Figure 6. Solubility of [^{14}C]-11β in MEM cell culture media with and without addition of 10% fetal calf serum (FCS).

In addition to hydrolysis of the reactive alkylating group, metabolic transformations occurring at other parts of the molecule could result in decreased affinity for the AR. Thus, knowledge of the pathways responsible for metabolism of 11β and pathways of excretion can provide

additional guidance for formulation or redesign of the compound to increase its efficacy and bioavailability. Phase I metabolism studies were conducted by incubating radiolabeled 11β with a microsomal fraction prepared from mouse liver. [^{14}C]-11β was incubated for 30 min at 37^{o}C with 0.25 mg/ml microsomal protein along with NADPH and an NADPH regenerating system consisting of glucose-6-phosphate dehydrogenase and glucose-6-phosphate. Products were extracted with ethyl acetate and analyzed by reversed-phase HPLC. Figure 7.**A** shows a radio-chromatogram obtained in the absence of NADPH, which is required for oxidase activity catalyzed by cytochrome P450s. The major peak at 28 min represents unchanged 11β. Several earlier eluting peaks likely represent products of spontaneous hydrolysis of the *N*,*N*-bis-chloroethyl group. Thus, in the absence of NADPH little metabolism occured. Figure 7.**B** shows that when NADPH was included a number of more polar compounds were rapidly formed. Analysis of several of the metabolic products by mass spectrometry indicated that they are primarily hydroxylated forms of the compound. These results have led to further studies to identify the molecular site(s) on the 11β molecule that is most vulnerable to such oxidations as well as the responsible P450 isoform(s). Preliminary results indicate that the steroid ring system is a major site of metabolism with formation of several hydroxylated species. The identification of sites that are susceptible to oxidation by P450s will permit us to redesign the molecule to bock or reduce metabolism which could result in an increased half life of the active molecule and greater therapeutic index by lowering the dose required to achieve an antitumor response. With the results we have obtained so far, we are poised to achieve these goals. Future work will identify the principle metabolites formed from 11β and also examine Phase II metabolism.

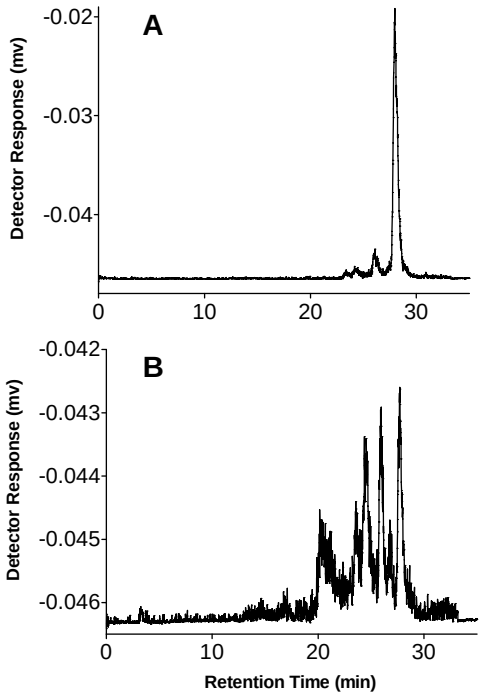

Figure 7. HPLC analysis of radiolabeled products formed by incubation of [^{14}C]-11β with mouse liver microsomes: A. without NADPH present; B. with NADPH.

Task 4: *Assess the antitumor activity of 11β in additional human prostate tumor models.*

In this Task we proposed to test the antitumor activity of 11β in several mouse xenograft models of human prostate cancer. Our objective was determine if the effectiveness of the 11β compound in preventing the growth of androgen-sensitive LNCaP cells in mouse xenografts was translatable to other tumor models that are representative of advanced androgen-independent forms of the disease. A major obstacle to our work has been the limited number and availability of well-characterized cell lines that express the AR but are androgen independent.

We first obtained a set of cell lines that we thought would be ideal for investigating the role of the AR in antitumor responses in xenograft models. These cell lines were genetically engineered derivatives of the PC3 cell line that were reportedly isogenic except for AR status. Our initial experiments were encouraging. We found that the PC3-AR cell line that expressed the AR had increased the sensitivity towards 11β compared to the control cell line PC3-Neo. Further evaluation of these lines, however, found that the PC3-AR cell line rapidly lost its ability to express the AR even when maintained under selective conditions. This situation was not ideal for our xenograft studies. Thus, we sought other prostate cancer cell models with stable expression of the AR that would permit us to expand our *in vivo* studies.

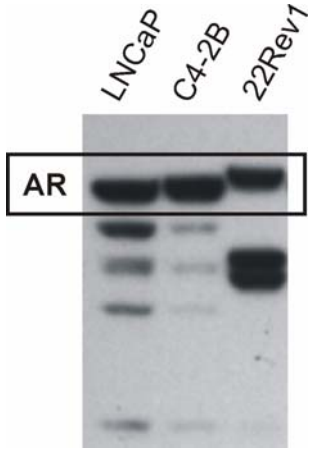

Figure 8. Western blot analysis of androgen receptor (AR) in prostate cancer cell lines. (equal amounts of protein loaded)

We have recently obtained several androgen-independent prostate cancer cell lines and characterized the sensitivity to 11β *in vitro*. Based on our experience with the PC3 lines we have been careful in validating these cell lines for xenograft models in our proposed work. One cell line we have tested is the C4-2B LNCaP subline which exhibits progressive tumorigenicity, metastatic potential and is androgen independent. We have also tested the 22Rev1 cell line which was established from a relapse of the transplantable human prostate tumor CWR22. The CWR22 cell line is androgen-dependent and xenograft tumors regresses in response to androgen withdrawal. The growth of 22Rev1 cells is androgen-independent. We first compared the levels of AR expression in the AR-independent cells lines with that of LNCaP cells. Figure 8 shows a Western analysis that we performed to compare the expression levels of AR in the three cell lines. We found similar AR levels in LNCaP and C4-2B cells. 22Rev1 cells expressed slightly lower levels of a higher molecular form of the AR which has been reported to contain a short insertion in the amino terminal end of the protein. We also confirmed that while the C4-2B and 22Rev1 cells express the AR, they are not sensitive to the AR antagonist bicalutamide indicating that they have characteristics of the androgen-independent form of advanced clinical disease.

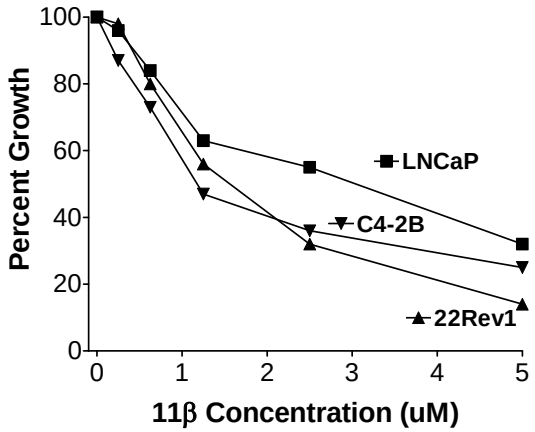

Figure 9. Toxicity of 11β towards AR+ prostate cancer cell lines.

Toxicity experiments were conducted by exposing cells in exponential growth phase to 11β and comparing cell number in treated and control populations after 48 h. These results indicate that the androgen independent C4-2B and 22Rev1 cells have greater sensitivity towards 11β compared to that of LNCaP cells. Based on these results we would predict that 11β would have greater efficacy in

preventing growth of C4-2B and 22Rev1 xenografts than we previously observed in the LNCaP model.

Thus, we now have relevant cell lines that represent advanced forms of prostate cancer for which we have characterized AR expression levels and their sensitivity towards 11β. Because of time and personnel resources we have not completed the proposed animal studies. We are seeking additional resources to carry out these crucial experiments.

KEY RESEARCH ACCOMPLISHMENTS

- We have characterized the toxicity of our novel antitumor compound 11β towards several prostate cancer cell lines and determined that the response of p21 is not useful as a potential biomarker of antitumor effects *in vivo*;
- We established the relationship between the level of 11β-DNA adducts formed in LNCaP cells in culture and cytotoxicity;
- We have assessed the oral bioavailability of 11β in mice and determined that our compound is not well absorbed when administered orally;
- We have determined that the 11β compound can be extensively metabolized by the P450 microsomal enzyme system and that the primary site of metabolism is the steroid ring system;
- We have developed methods to examine both the total level of 11β-DNA adducts formed in cells and tissues as well as individual species of 11β adducts that may be better correlated with the cytotoxic and therapeutic effects of this compound;
- We have characterize androgen receptor levels and sensitivity towards 11β of several prostate cancer cell lines that are models of androgen-independent prostate cancer. These cell lines will provide a crucial test of the potential for 11β to prevent the growth of advanced forms of prostate cancer in animal models.

REPORTABLE OUTCOMES

Publications: Hillier, S.M., Marquis, J.C., Zayas, B., Wishnok, J.S., Liberman, R.G., Skipper, P.L., Tannenbaum, S.R., Essigmann, and Croy, R.G. DNA adducts formed by a novel antitumor agent 11β-dichloro in vitro and in vivo. 2006 Mol. Cancer Ther. 5(4); 977-984.

Personnel Supported

Dr. Robert Croy was supported on this grant. He provided supervision and coordination of the research.

Dr. Alfio Fichera has received support from this grant. He is a Postdoctoral scientist with a Ph.D. in Chemistry. He synthesized the molecules described in this project.

Mr. Frances Gonzales is a graduate student who has been supported on this grant. He received a Masters Degree from MIT.

Ms. Sreeja Gopal, a graduate student has been supported with funds from this grant. She is investigating the kinetics of 11β-DNA adducts in cell cultures and animal models.

CONCLUSIONS

The Specific Aims of our PCRP Idea Development Award focused on the development of DNA damaging warheads tethered to ligands for the androgen receptor, which is over expressed in most prostate cancers. Out of that work we prepared a compound, 11β, which has become the focus of our work over the past three years. As originally proposed, our efforts have focused on connecting the unique biochemical responses to 11β that we have observed in cell culture to its antitumor effects in xenograft mouse models. We also wanted to determine if some of these responses could serve as biomarkers of tumor responses that would be of potential use for human clinical trials.

Several cellular responses to 11β – such as increased levels of the p21 protein – were investigated to determine if the changes in protein levels we discovered in cells in culture could also be observed in tumors in xenograft animal models. We found this impractical with the available techniques because of the heterogenous nature of the cell populations in these tumors. Fortunately, we found in our further investigations that the formation and fate of 11β-DNA adducts in cells and tissues had the potential to serve as biomarkers of both exposure and response in normal and tumor tissues. We therefore refocused a portion of our effort for this Task on the development of the sensitive methods required for 11β adduct analyses.

Two extremely sensitive and quantitative methods for the analysis of 11β-DNA adducts have been developed. The first method takes advantage of a radiolabelled analog of 11β that we synthesized containing one [^{14}C] atom. Using the radiolabelled compound we developed a highly sensitive method for adduct analysis that is based on the technique of Accelerator Mass Spectrometry (AMS). The AMS analysis enabled us to quantify the overall level of DNA damage in both cells in culture following exposure to 11β and the amounts of adducts formed in xenograft tumors in animals after treatment. We found that the levels of 11β-DNA adducts in tumor tissues were within the range of those we observed in cells in culture treated with cytotoxic levels of 11β. This result suggests that the biochemical responses we identified *in vitro* have relevance to the antitumor effects of 11β in animals.

The second method we developed for analysis of 11β-DNA adducts is based on HPLC-ESI$^+$-MS/MS. This technique permits quantification of individual adduct species including monoadducts and crosslinks which we believe are essential for antitumor effects. To enable analysis of adducts from DNA isolated from cells and tissues we developed a solid phase extraction (SPE) protocol using a mixed weak cation exchange and reversed phase sorbent. Using these techniques we identified guanine and adenine 11β-monoadducts and bis-guanine 11β crosslinks in prostate cancer cells treated in culture. We are now at the point of being able to test whether these methods have the sensitivity required for the analysis of individual 11β-DNA adducts in tumors growing as xenografts following treatment. If successful these methods will provide a means to directly measure doses received by the tumor and greatly facilitate the development of formulations and dosage regimens for preclinical and clinical studies.

We also conducted a pharmacokinetic and metabolism studies on 11β to determine if it was possible to administer the compound orally. These experiments found very low levels of absorption of 11β via the gut. As a result, our further formulation studies will be directed to the development of a vehicle that can be administered *via* i.v. injection. Metabolism of 11β was explored using microsomal fractions containing P450s from mouse liver. We discovered a number of oxidized metabolites of 11β which were rapidly formed by our *in vitro* system. Preliminary data indicate that some of these metabolites are formed by hydroxylations occurring on the steroid moiety. If these modifications decrease affinity for the AR they could have adversely affect the antitumor activity of 11β. We are conducting additional structural studies on these metabolites to determine the exact location of the oxidation and its consequences on AR binding. This information could lead to the development of analogs that are less susceptible to metabolism and consequently have greater antitumor efficacy.

Our last area of progress has been investigation of the potent antitumor activity of 11β in additional xenograft animal models of prostate cancers. We previously showed that at tolerable doses the 11β is a very effective inhibitor of the growth of LNCaP xenograft tumors in mice. Initially, we obtained a pair of cell lines derived from the AR-negative PC3 human prostate cancer cell line that had been transfected with either an AR expression vector (PC3-AR) or a control vector (PC3-Neo). The goal of these studies was to establish xenograft tumors from these cell lines and determine the role played by the AR in the antitumor effects of 11β. In characterization of these cell lines *in vitro*, however, we discovered that levels of AR in the PC3-AR line were unstable and decreased significantly over time even when the cells were maintained under selective conditions. We concluded that these cell lines were not appropriate for our proposed studies. We then obtained several other human prostate cancer cell lines which we have characterized with respect to their sensitivity towards 11β and AR expression levels. Both cell lines we are now working with (C4-2B and 22Rev1) have stable levels of AR expression even though they are androgen independent for growth. Thus, these cell lines represent advanced hormone-refractory stages of prostate cancer. We are now at the point of initiating the animal studies to complete this work.

Prostate tumors are typically refractory to apoptosis induced by DNA damaging agents. By tethering a DNA damaging warhead to a ligand for the androgen receptor, we have designed a molecule that forms DNA adducts that interact with the androgen receptor. These lesions efficiently kill prostate cancer cells in culture by apoptosis, and inhibit tumor growth *in vivo*. These molecules afford a new approach to the treatment of clinically advanced forms of prostate cancer. The investigations conducted during the 3-year grant period have provided key information that will permit us to optimize the antitumor effects of 11β and conduct further preclinical and clinical studies.

REFERENCES

None included.

APPENDICIES

1. Copy of Hillier et al. 2006 Mol. Cancer Res. 5, 977- 984.

DNA adducts formed by a novel antitumor agent 11β-dichloro *in vitro* and *in vivo*

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Abstract

The multifunctional molecule 11β-dichloro consists of a ligand for the androgen receptor linked to a bifunctional alkylating group, permitting it to create DNA adducts that bind the androgen receptor. We propose that binding of the androgen receptor to 11β-DNA adducts acts to both shield damaged sites from repair and disrupt the expression of genes essential for growth and survival. We investigated the formation 11β-DNA adducts in tumor xenograft and nontumor tissues in mice. Using [^{14}C]-11β-dichloro, we show that the molecule remains intact in blood and is widely distributed in mouse tissues after i.p. injection. Covalent 11β-guanine adducts identified in DNA that had been allowed to react with 11β-dichloro *in vitro* were also found in DNA isolated from cells in culture treated with 11β-dichloro as well as in DNA isolated from liver and tumor tissues of mice treated with the compound. We used accelerator mass spectrometry to determine the levels of [^{14}C]-11β-DNA adducts in LNCaP cells treated in culture as well as in liver tissue and LNCaP xenograft tumors in treated mice. The level of DNA adducts in tumor tissue was found to be similar to that found in LNCaP cells in culture treated with 2.5 μmol/L 11β-dichloro. Our results indicate that 11β-dichloro has sufficient stability to enter the circulation, penetrate tissues, and form DNA adducts that are capable of binding the androgen receptor in target tissues *in vivo*. These data suggest the involvement of our novel mechanisms in the antitumor effects of 11β-dichloro. [Mol Cancer Ther 2006;5(4):977–84]

Introduction

Prostate cancer is the second most commonly diagnosed cancer and the fourth leading cause of cancer death among men in developed countries (1). Most prostate cancers depend on androgens for their growth. Therefore, chemical or surgical castration and/or treatment with hormonal antagonists is often given as adjuvant therapy following the surgical removal of the tumor. Androgen ablation results in the apoptotic death of androgen-sensitive cells producing an initial therapeutic response (2–5). Unfortunately, patients who undergo these adjuvant treatments often develop aggressive and metastatic androgen-independent forms of hormone-refractory prostate cancer. New and more effective drugs are needed to treat advanced stages of this disease, as well as to treat more effectively its earlier and less aggressive forms.

The androgen receptor is expressed throughout the development of prostate cancer and is present in most patients with hormone-refractory prostate cancer (6–9). Clinical evidence suggests that disruption of androgen receptor signaling through dysregulation of androgen receptor coregulators, androgen receptor gene amplification, or mutations in the androgen receptor enables it to remain transciptionally active in the presence of androgen receptor antagonists and other therapeutics (10, 11). The continued role of the androgen receptor in hormone-refractory prostate cancer provides an attractive target for therapeutic development.

We have recently reported the development of a novel cytotoxic agent that was designed to take advantage of the dysregulation of the androgen receptor in hormone-refractory prostate cancer (12). 11β-Dichloro is a bifunctional compound in which an androgen receptor ligand is linked to a p-*N*,*N*-bis-(2-chloroethyl)aminophenyl moiety that can produce covalent damage to DNA. A consequence of the stable connection between the groups at either end of the 11β-dichloro molecule is that the androgen receptor can bind to covalent adducts that are formed in DNA. It is hypothesized that these androgen receptor-DNA adduct complexes both shield the DNA adduct from repair enzymes as well as prevent the androgen receptor from acting (by its normal function) to promote cell growth and survival (Fig. 1). This latter mechanism, inactivation of the androgen receptor through its physical association with DNA adducts, is different from the mechanism of currently used antagonists.

The 11β-dichloro compound rapidly induces apoptosis in LNCaP cells in culture at concentrations >5 μmol/L and is highly effective in preventing the growth of LNCaP tumors

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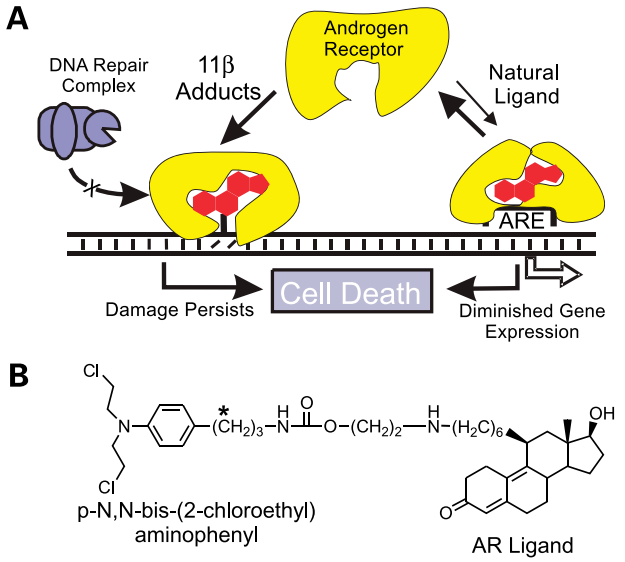

Figure 1. A, scheme illustrating the proposed effects of 11β-DNA adducts on androgen receptor function and DNA repair in target cells. The steroid ligand portion of 11β adducts could form a complex with the androgen receptor that prevents recognition and removal by the DNA repair complex (*left*). 11β-DNA adducts compete with the natural ligand for the androgen receptor (DHT) and antagonize its transcriptional function, leading to diminished gene expression (*right*). The combination of these mechanisms results in apoptosis and cell death. **B,** structure of 11β-dichloro; *, position of ^{14}C atom.

as xenografts in mice (90% growth inhibition; ref. 12). The studies described here were designed to examine the tissue distribution of 11β-dichloro in mice and to determine whether the intact molecule is capable of forming DNA adducts *in vivo* that are required for our proposed mechanisms of action (i.e., adducts capable of interacting with the androgen receptor). We have also used the highly sensitive technique of accelerator mass spectrometry to investigate the formation of 11β-DNA adducts in a human prostate tumor growing as s.c. xenografts in mice.

Materials and Methods

Chemicals

[^{14}C]-11β-Dichloro, (3-{4-[bis-(2-chloro-ethyl)-amino]-phenyl}-3-[^{14}C]propyl)-carbamic acid 2-[6-(17-hydroxy-13-methyl-3-oxo-2,3,6,7,8,11,12,13,14,15,16,17-dodecahydro-1*H*-cyclopenta[*a*]phenanthren-11-yl)-hexylamino]-ethyl ester, was synthesized with one ^{14}C atom in the propyl group connecting the carbamate and the p-*N*,*N*-bis-(2-chloroethyl)aminophenyl moieties using previously described procedures (13). The position of the radiolabel is indicated in Fig. 1B. Initial radiochemical purity was >98% and specific activity was 50 mCi/mmol. Unlabeled 11β-dichloro was used to dilute the radiolabeled material where necessary.

Animals

Four- to six-week-old NIH Swiss Webster mice were purchased from Charles River Laboratories (Wilmington, MA). Female athymic NIH Swiss (*nu/nu*) mice (25 g) were obtained from the National Cancer Institute, Frederick Cancer Center (Frederick, MD). All experiments were done under the guidelines of the Massachusetts Institute of Technology Animal Care Committee.

Dose Formulation

[^{14}C]-11β-Dichloro or unlabeled 11β-dichloro dissolved in a small volume of ethanol was added to cremophor-EL/saline (final proportions, cremophor-EL 37%/ethanol 27%/saline 30%) for administration by i.p. injection.

Biodistribution Analysis

NIH Swiss Webster mice received a single dose of 45 mg/kg of [^{14}C]-11β-dichloro (specific activity, 1.91 mCi/mmol) in 50 μL of vehicle administered via i.p. injection. Animals were sacrificed by carbon dioxide asphyxiation at 0.25, 1, 2, 4, 6, and 24 hours postinjection; blood samples obtained by cardiac puncture were collected in heparinized syringes; and the following tissues were excised from each animal: lung, liver, spleen, kidney, intestine, and in some instances adipose, heart, and skeletal muscle.

Intestinal contents were also collected. Tissue samples were flash-frozen and stored at −20°C before processing. Approximately 100 mg of each tissue (10 μL of whole blood) were placed in preweighed vials and solubilized in 1 mL of Solvable (Packard Biosciences, Meriden, CT) heated to 65°C for 3 hours or until the tissue was completely dissolved. The solutions were decolorized by treatment with 200 μL of 30% hydrogen peroxide. After addition of 15 mL Hionic Fluor scintillation fluid (Packard Biosciences), radioactivity was determined using a Beckman LS1801 Liquid Scintillation Counter.

Analysis of 11β-Dichloro in Blood

Two volumes of acetonitrile were added to whole blood and the precipitate was isolated by centrifugation (5 minutes; 13,000 × *g*). The amount of 11β-dichloro covalently bound to proteins was assessed by determining the ^{14}C activity in aliquots of the supernatant (organic soluble phase) and precipitate.

The precipitated material was solubilized with 1 mL of Solvable (Packard Biosciences), heated overnight at 70°C, and decolorized by addition of 200 μL 30% hydrogen peroxide. The fraction of noncovalently bound 11β-dichloro equals the amount of ^{14}C in the organic soluble phase divided by the total amount of ^{14}C in blood.

The amount of intact 11β-dichloro that was present in blood was determined by high-performance liquid chromatography analysis. An aliquot of the organic soluble phase (supernatant) was dried in a Savant SC-110 SpeedVac, dissolved in 100 μL acetonitrile, and injected onto a Beckman octadecyl silane 4.6 × 250 mm Ultrasphere column at a flow rate of 1 mL/min with 10% CH_3CN, 50% methanol containing 0.1 mol/L ammonium acetate. Compounds were eluted from the column with a 20-minute linear gradient that increased the concentration of methanol to 100%. Compounds were detected by a tandem configuration of UV (Rainin UV-1 UV Detector) and radiochemical (Packard Flow Scintillation Analyzer Model

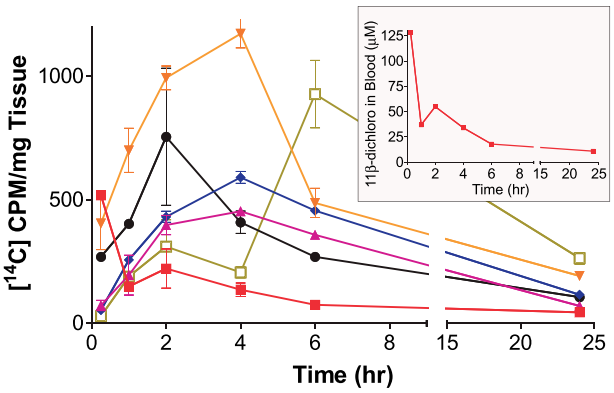

Figure 2. Distribution of [^{14}C]-11β-dichloro in blood and selected tissues. NIH Swiss Webster mice were administered 45 mg/kg [^{14}C]-11β-dichloro (specific activity, 1.91 mCi/mmol) via i.p. injection and sacrificed at the indicated time points. Tissues were solubilized and radioactivity measured by scintillation counting (n = 3; *bars,* SD). ■, blood; ▼, liver; ▲, lung; ♦, kidney; ●, intestine; □, feces. *Inset,* concentration of 11β-dichloro in blood.

150TR) detectors. The radiochemical detector was calibrated with a known amount of [^{14}C]-11β-dichloro that was used to correlate peak area with the amount of [^{14}C]-11β-dichloro in the sample.

Reaction of 11β-Dichloro with DNA *In vitro* and Identification of Covalent Products

Salmon testes DNA was dissolved in 5 mmol/L sodium cacodylate and *N,N*-dimethylformamide was added to a final concentration of 25%. 11β-Dicholoro was added in DMSO (50 μmol/L) and the solution incubated for 16 hours at 37°C. After phenol/chloroform extraction, the DNA was isolated by ethanol precipitation. Covalent products were released by acid hydrolysis in 0.1 N HCl (30 minutes, 70°C); after which, the solution was neutralized with NaOH and adjusted to 20 mmol/L Tris-HCl (pH 7.4) and 10% methanol. The solution was then loaded directly onto a C18 SepPak column (Waters Co., Milford, MA) that was sequentially eluted with 10%, 50% aqueous methanol solutions, and finally 100% methanol. After reducing the volume of the 100% methanol fraction, an aliquot was analyzed by high-performance liquid chromatography using conditions described above. Compounds in fractions collected from the high-performance liquid chromatography were analyzed by electrospray ionization mass spectrometry on an Agilent 1100 Series LC/MSD Trap operated in the positive ion mode. Samples were introduced by flow injection (0.2 mL/min) in methanol/10 mmol/L NH_4Ac in H_2O/acetonitrile (50:45:5).

Identification of 11β-Dichloro DNA Adducts *In vivo*

NIH Swiss Webster mice were treated with 11β-dichloro (25 mg/kg, i.p.) and sacrificed 4 hours later by asphyxiation with 95% CO_2. Livers were removed surgically, snap frozen on dry ice, and stored at −80°C. Thawed tissue was minced and homogenized (Dounce) in 3 volumes of cold 0.01 mol/L Tris-HCl (pH 6.9), 0.25 mol/L sucrose, 2 mmol/L calcium chloride (lysis buffer). After filtering through nylon mesh, Triton X-100 was added to a final concentration of 5% and a crude nuclear fraction collected by centrifugation at 1,000 × g for 20 minutes at 4°C. The pellet was resuspended in 2 volumes of lysis buffer, to which SDS and sodium chloride were added to final concentrations of 1% and 1 mol/L, respectively. The viscous solution was extracted twice with chloroform/isoamyl alcohol (24:1) and nucleic acids collected by ethanol precipitation.

RNA was removed by digestion with RNase A. The isolated nucleic acids were dissolved in 2 mL of 0.05 mol/L Tris-HCl (pH 7.5), 0.1 mol/L NaCl and treated with 0.5 mg RNase A (10 minutes; 37°C). Following extraction with chloroform/isoamyl alcohol (24:1), DNA was isolated by ethanol precipitation. The DNA was then subjected to acid hydrolysis and released products were analyzed by high-performance liquid chromatography and electrospray ionization mass spectrometry as described above.

Cell Culture

LNCaP cells were obtained from the American Type Culture Collection (Rockville, MD) and maintained in RPMI 1640 (Life Technologies, Inc., Carlsbad, CA) supplemented with 2.5 mg/mL glucose, 1 mmol/L sodium pyruvate, 100 mmol/L HEPES, 2 mmol/L glutamax, and 10% fetal bovine serum in a humidified 5% CO_2/air atmosphere at 37°C. LNCaP cells were seeded in six-well plates and allowed to attach to the surface for 24 hours. Cells were then exposed to the indicated dose of [^{14}C]-11β-dichloro dissolved in DMSO for the indicated period of time. At the end of the incubation, the cells were harvested by trypsinization, pelleted, and washed with PBS. The DNA from the cellular pellet was isolated according to the procedure described above.

For growth inhibition experiments, LNCaP cells were seeded in six-well dishes at 10^5 per well. Forty-eight hours later, the test compound was added in DMSO solution. Cells were detached by trypsin/EDTA after 36 hours and the number of cells in control and 11β-dichloro-treated wells determined using a Coulter Counter. The percent growth inhibition is the ratio of cell number in treated and control wells multiplied by 100.

^{14}C Accelerator Mass Spectrometry Analysis of DNA Adducts

Accelerator mass spectrometry analyses were conducted by the Biological Engineering Accelerator Mass Spectrometry Lab at Massachusetts Institute of Technology as

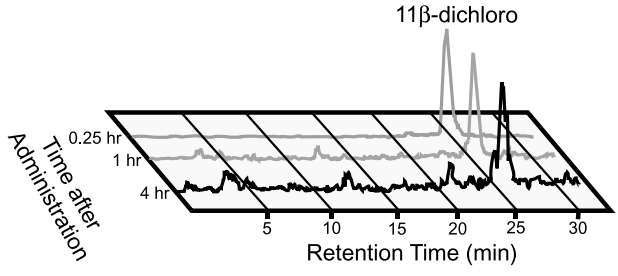

Figure 3. Reversed phase high-performance liquid chromatography analysis of radiolabeled compounds isolated from blood of mice at 0.25, 1, and 4 h after administration of [^{14}C]-11β-dichloro. The peak labeled 11β-dichloro represents the intact compound. For chromatographic conditions, see Materials and Methods.

described in detail elsewhere (14). DNA concentrations were determined by UV absorption at 260 nm using a Beckman DU-65 UV-Vis Spectrophotometer. Solutions of DNA dissolved in water were applied directly to a CuO matrix used for sample combustion. A standard consisting of a solution of [^{14}C-methyl]-bovine serum albumin was used to calibrate the instrument. The amount of ^{14}C in each sample was calculated from the peak area ratio of the sample to the standard. All samples were analyzed at least twice.

Tumor Implantation and Formation of 11β-Dichloro DNA Adducts in Xenografts

Tumor xenografts were established on the flank of NIH Swiss *nu/nu* mice by s.c. injection of 2×10^6 LNCaP cells suspended in 0.25 mL of serum-free medium/Matrigel (1:1; Becton Dickinson, Franklin Lakes, NJ). When the tumors reached ~300 mm^3 (5-6 weeks), animals received an i.p. injection of 50 mg/kg of [^{14}C]-11β-dichloro (specific activity, 0.5 mCi/mmol). Four hours later, DNA was isolated as described above from liver and tumor tissues of individual mice and subjected to accelerator mass spectrometry analysis to determine the amount of covalently bound radioactivity.

Analysis of Liver Toxicity in Mice

NIH Swiss *nu/nu* mice injected with either single or repeated dose(s) of 11β-dichloro were sacrificed by carbon dioxide asphyxiation. Blood was collected by cardiac puncture and placed in a Becton Dickinson Microtainer Serum Separator tube for blood chemistry analysis, which was done by IDEXX Laboratories (North Grafton, MA).

Results

Distribution of Radioactivity in Blood and Selected Tissues

[^{14}C]-11β-Dichloro was rapidly absorbed into the circulation following i.p. injection. The peak concentration of radiolabeled 11β-dichloro in blood of 128 μmol/L was found at 15 minutes and declined rapidly as shown in Fig. 2 (*inset*) with a half life ($t_{1/2}$) of 1.3 hours. [^{14}C]-11β-Dichloro was well distributed into the tissues (Fig. 2). At 15 minutes after injection, the highest levels of radioactivity were found in the liver, intestine, spleen, and lung. High levels of radioactivity were also found in fat (not shown). Tissue concentrations reached maximum levels at 4 hours with the liver and kidney experiencing the highest concentrations. After 4 hours, there was rapid accumulation of radioactivity in feces, which is consistent with biliary excretion as a major route of 11β-dichloro elimination.

Following organic extraction, <10% of radioactivity in blood remained associated with precipitated proteins, consistent with a low level of covalent modification of blood proteins. High-performance liquid chromatography analysis found that >90% of the organic-soluble radioactivity in blood after 1 hour corresponded to the intact 11β-dichloro compound (Fig. 3). At 4 hours, several earlier eluting compounds were observed, which are probably 11β-dichloro metabolites (Fig. 3). The majority of the radioactivity (>65%), however, still corresponded to the parent compound.

Identification of 11β-DNA Adducts *In vitro*

The presence of the parent 11β-dichloro compound in blood indicated that the intact molecule could be distributed to tissues and was available to react with cellular DNA forming covalent adducts. A key question about the proposed mechanism of action of the new compound is whether the intact molecule is capable of forming DNA adducts *in vivo*. To answer this question, we first characterized the DNA adducts formed by the reaction of 11β-dichloro with DNA *in vitro*. Salmon testis DNA that had been incubated with 11β-dichloro was subjected to acid hydrolysis and the hydrolyzed material was analyzed by reversed phase high-performance liquid chromatography.

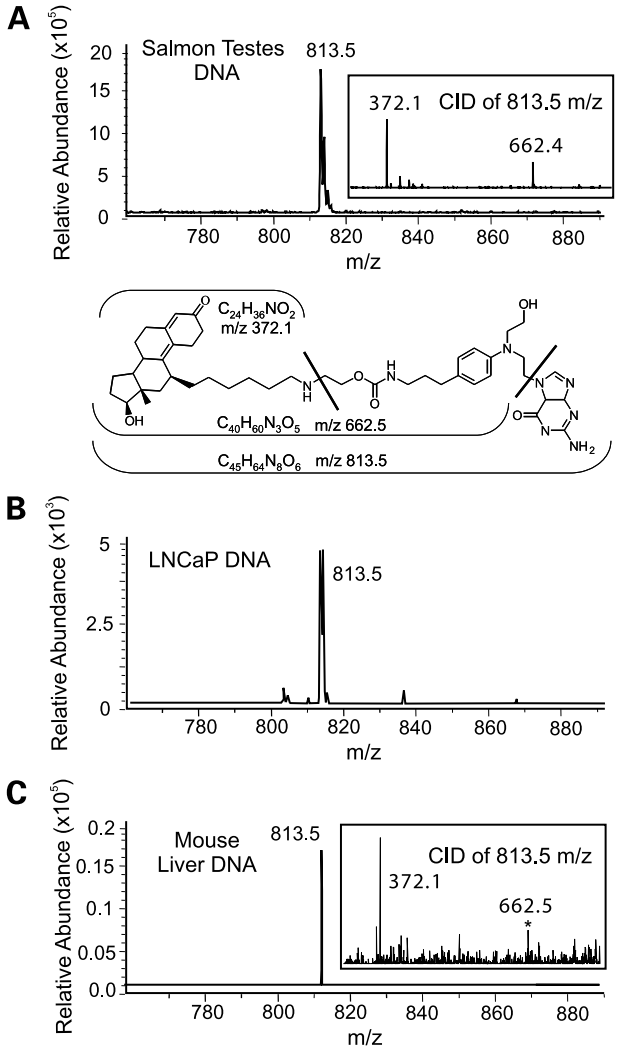

Figure 4. Electrospray ionization mass spectrometry analysis of 11β-dichloro-DNA adducts formed *in vitro* and *in vivo*. **A,** salmon testes DNA reacted with 11β-dichloro *in vitro*; **B,** DNA obtained from LNCaP cells in culture treated with 10 μmol/L 11β-dichloro for 6 h; **C,** DNA isolated from liver tissue of NIH Swiss *nu/nu* mice treated with 25 mg/kg (i.p.) 11β-dichloro. Proposed structures of the most abundant ion (813.5 *m/z*) and collision-induced dissociation (*CID*) fragments (*insets*) are shown in **A**. DNA was subjected to hydrolysis with 0.1 N HCl to release covalent adducts (see Materials and Methods).

A single peak was observed that eluted before the unreacted 11β-dichloro compound. Analysis of the eluted material by electrospray ionization mass spectrometry identified an ion with 813.5 *m/z* (Fig. 4A) corresponding to $[M + H]^+$. This ion is consistent with a chemical structure in which one ethylene group at the aniline nitrogen of the 11β molecule is attached to a guanine base whereas the other ethylene has a hydroxyl group substituted for chlorine (Fig. 4A, *bottom*). We did not investigate whether hydrolysis of the chlorine occurred during reaction with DNA or at a subsequent step in our analytic procedure. Further analysis of the 813.4 *m/z* molecular ion by collision-induced dissociation yielded prominent fragment ions at *m/z* 662.4 and *m/z* 372.1 (Fig. 4A, *inset*). The proposed structure of the guanine adduct formed by 11β-dichloro has a parent ion and collision-induced dissociation fragments analogous to those produced by a DNA adduct formed by a structurally related compound E2-7α in which estradiol is linked to the reactive p-*N,N*-bis-(2-chloroethyl)aminophenyl group (13). It is likely that the 11β molecule forms a covalent bond at the N7 atom of guanine but we did not have sufficient material to allow complete structural characterization.

Analysis of 11β-DNA Adducts in LNCaP Cells in Culture

Following preliminary characterization of the 11β-DNA adduct formed *in vitro*, we investigated whether the same adduct was formed in LNCaP cells in culture. The 11β-dichloro compound rapidly induces apoptosis in this androgen receptor–expressing human prostate cancer cell line (12). We isolated DNA from LNCaP cells after treatment with 10 μmol/L 11β-dichloro for 6 hours, which was then hydrolyzed using the same conditions used for *in vitro* modified DNA. The hydrolyzed products were then analyzed by electrospray ionization mass spectrometry. Among the hydrolysis products was a prominent ion at *m/z* 813.5, which was identical to the product identified in DNA that was directly reacted with 11β-dichloro *in vitro* (Fig. 4B). Thus, the 11β-dichloro molecule remains intact under cell culture conditions and reacts with guanine bases in cellular DNA. There were no marked differences between the DNA adduct that was identified *in vitro* and the one formed in LNCaP cells in culture.

We next investigated the formation of covalent 11β-DNA adducts in LNCaP cells by treating them with [^{14}C]-11β-dichloro. DNA isolated from treated cells was analyzed by accelerator mass spectrometry to determine the amount of covalently bound [^{14}C]-11β-dichloro. The high sensitivity of the accelerator mass spectrometry technique permitted us to detect and quantify the levels of covalently bound ^{14}C that were present in cellular DNA. We first established a dose-response relationship for adduct formation by treating LNCaP cells with 2.5, 5, or 10 μmol/L [^{14}C]-11β-dichloro for 4 hours. The amount of ^{14}C per microgram of DNA increased in direct proportion with 11β-dichloro concentration in the growth media (Fig. 5A). Based on specific activity, the level of 11β-DNA adducts rose from 0.3 to 1.0 adducts per 10^6 bases over the dose range of 2.5 to 10 μmol/L 11β.

We also investigated the rate of formation of 11β-DNA adducts over a 15-hour period in LNCaP cells treated with a single dose of 10 μmol/L [^{14}C]-11β-dichloro. Figure 5B shows that the concentration of 11β-DNA adducts in these cells increased at a constant rate during the 15-hour period. The slope of the line in Fig. 5B implies a rate of adduct formation of 0.25 adducts/10^6 bases per hour. This rate of adduct formation correlates well with the observed number of adducts present in LNCaP cells after 4-hour exposure to 10 μmol/L 11β-dichloro (Fig. 5A). These results are also consistent with the stability of the 11β-dichloro compound in cell culture media because rapid destruction of the compound by hydrolysis or metabolism would be expected to decrease the rate of 11β adduct formation.

Figure 5C shows the dose-response relationship for growth inhibition of LNCaP cells by 11β-dichloro. The ED_{50} for growth inhibition calculated from these data is 5.3 μmol/L.

Identification of 11β-DNA Adducts in Liver and Xenograft Tumor Tissue

The effectiveness of 11β-dichloro in inhibiting the growth of LNCaP prostate tumor cells growing as s.c. xenografts in mice (12) led us to examine the formation of 11β-DNA adducts in xenograft and normal tissues. First, to establish the identity of adducts that were formed in tissues of mice

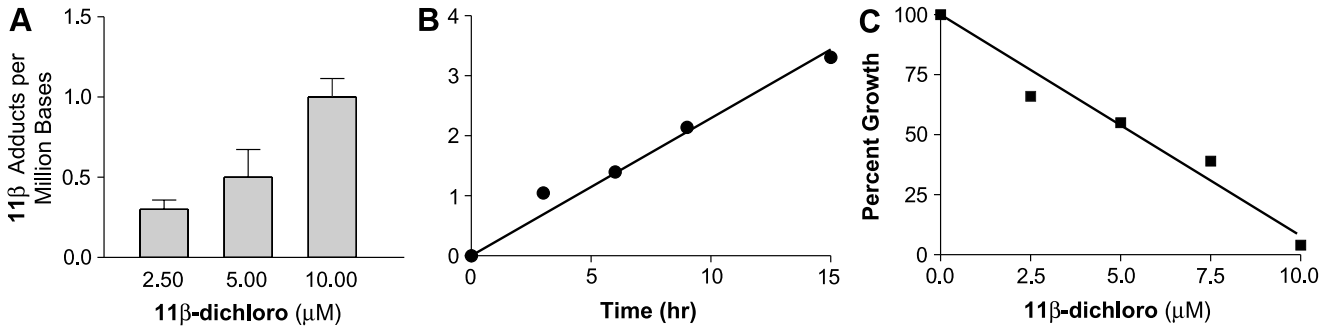

Figure 5. Formation of 11β-DNA adducts and inhibition of LNCaP cell growth by 11β-dichloro. **A,** relationship between 11β-dichloro concentration in growth media and level of 11β-DNA adducts formed after 4-h exposure. **B,** rate of formation of 11β-DNA adducts in LNCaP cells exposed to 10 μmol/L 11β-dichloro. **C,** growth inhibition of LNCaP cells by 11β-dichloro (*bars,* SD).

Table 1. [^{14}C] accelerator mass spectrometry analysis of 11β-DNA adducts in liver tissue and LNCaP tumor xenografts

Mouse	Liver		LNCaP xenograft (i.p.)	
	amol ^{14}C/μg DNA	11β adducts/10^6 DNA bases	amol ^{14}C/μg DNA	11β adducts/10^6 DNA bases
1	51 ± 5	1.7 ± 0.2	4.1 ± 1.4	0.14 ± 0.05
2	52 ± 9	1.7 ± 0.3	6.3 ± 0.7	0.21 ± 0.02
3	45 ± 5	1.5 ± 0.2	7.3 ± 0.7	0.24 ± 0.02

NOTE: Tissues were obtained from NIH Swiss *nu/nu* mice 4 hours after i.p. administration of 50 mg/kg [^{14}C]-11β-dichloro (specific activity, 0.5 mCi/mmol). DNA concentration was estimated by UV absorption. The adduct concentration in DNA (adducts per 10^6 bases) was calculated based on the amol ^{14}C/μg DNA determined by accelerator mass spectrometry analysis of the DNA. Results are mean ± SD of DNA samples isolated from mice (n = 3).

treated with 11β-dichloro, we investigated the presence of 11β-DNA adducts in liver because this tissue is exposed to the highest concentration of 11β-dichloro (Fig. 2). DNA was isolated from liver tissue of mice 4 hours after administration of 11β-dichloro (45 mg/kg, i.p.), hydrolyzed, and analyzed directly by electrospray ionization mass spectrometry. As in the case of studies in LNCaP cells in culture, we searched for molecular ions of ≥600 *m/z* and found one abundant ion of 813.5 *m/z* (Fig. 4C). Further analysis of this ion by collision-induced dissociation also produced fragment ions at *m/z* 662.5 and *m/z* 372.1. Thus, the DNA adduct formed by direct reaction of 11β-dichloro with DNA *in vitro* is identical by mass spectrometry to the adduct formed in DNA of cells in culture and in tissues *in vivo*. The fact that the intact 11β molecule is covalently linked to liver DNA indicates that the molecule has sufficient stability *in vivo* for distribution and penetration into tissues and, hence, is available to do its intended biological functions.

Having evidenced formation of DNA adducts by intact 11β-dichloro *in vivo*, we proceeded to investigate tumor concentrations of the 11β adducts in an animal xenograft model. Mice bearing xenograft LNCaP prostate tumors were administered a single dose of 50 mg/kg [^{14}C]-11β-dichloro (specific activity, 0.5 mCi/mmol). After 4 hours, samples of tumor and liver tissues were obtained and DNA was isolated and subjected to ^{14}C accelerator mass spectrometry analysis as described above. The results of these analyses are presented in Table 1. Based on the number of amol ^{14}C/μg DNA, tumor tissue had ~12% of the concentration of 11β-DNA adducts as were found in the liver (Table 1). Because accelerator mass spectrometry analysis cannot reveal the identity of the radiolabeled species, it will require further investigation to confirm that the results of our analyses represent the presence of the 11β-guanine adduct in tumor tissues. Nonetheless, these data ascertain the ability of 11β-dichloro to react with one of its intended molecular targets (DNA) in tumor tissue.

Investigation of Hepatotoxic Effects of 11β-Dichloro

An animal xenograft tumor study conducted to investigate the efficacy of 11β-dichloro against prostate cancer found little overt evidence of toxicity throughout a 45-day seven-course regimen using a dose of 30 mg/kg (12). Because 11β-dichloro concentrations were highest in the liver and this tissue was also found to experience higher levels of DNA damage than tumor tissue, we investigated whether four serum markers of hepatotoxicity were elevated after single or multiple doses of 11β-dichloro. In the case of acute exposures, blood was collected from NIH Swiss *nu/nu* mice 24 hours after they had received a single i.p. dose of 10, 30, 50, or 75 mg/kg 11β-dichloro. Blood was also collected from NIH Swiss *nu/nu* mice that had been treated repeatedly with 30 mg/kg 11β-dichloro on the same regimen used in the xenograft tumor study (12). Serum levels of four enzymes that are frequently elevated in drug toxicity were measured in treated animals and compared with levels in animals administered vehicle only as well as those of untreated historical controls.

Table 2. Serum enzyme levels in mice after single or repeated administration of 11β-dichloro

	Single dose (mg/kg)					Repeated dosing (mg/kg)	
	10	30	50	75	Vehicle	30	Vehicle
ALT	50 ± 24	47 ± 19	509 ± 205*	1,220 ± 960*	41 ± 5	36 ± 10	48 ± 10
AST	201 ± 153	105 ± 35	594 ± 181*	678 ± 540*	99 ± 49	112 ± 46	119 ± 33
GGT	<3 ± 1.7	0 ± 0	<3 ± 1.7	<3 ± 1.7	<3 ± 1.7	1.8 ± 0.5	1 ± 1
Alk phos	72 ± 13	77 ± 10	59 ± 14	69 ± 12	90 ± 12	58 ± 18	60 ± 5

NOTE: Enzyme levels were analyzed in whole blood collected by cardiac puncture 24 hours after single doses or from animals after completion of a 7-week protocol, during which they received five consecutive daily doses per week. Results are shown as average ± SD (n = 3).
Abbreviations: AST, aspartate aminotransferase; ALT, alanine aminotransferase; GGT, γ-glutamyl transferase; Alk phos, alkaline phosphatase.
*$P < 0.05$, Student's t test.

Increased levels of alanine aminotransferase and aspartate aminotransferase were found in animals treated with ≥50 mg/kg 11β-dichloro. After a single administration, we observed no changes in either γ-glutamyl transferase or alkaline phosphatase at doses up to 75 mg/kg 11β-dichloro (Table 2) whereas alanine aminotransferase and aspartate aminotransferase levels were elevated from 35- to 40-fold at this dose. No significant changes in the serum markers of liver toxicity were found in mice treated with 10 or 30 mg/kg. Furthermore, normal levels of all four serum enzymes were found in animals that had received repeated doses during seven courses of five daily doses of 30 mg/kg (Table 2).

We also examined blood reticulocytes as a measure of bone marrow toxicity in mice treated for an extended period of time with the 30 mg/kg dose. The average level of reticulocytes in treated mice ($6.9 \times 10^6/mm^3$; SD, 2.3) was not statistically different from vehicle-treated mice ($7.5 \times 10^6/mm^3$; SD, 1.3). Taken together, these data show that 11β-dichloro has acceptable toxicity at the level shown to effectively inhibit tumor growth.

Discussion

In this study, we have identified a covalent DNA adduct formed in cells in culture and tissues of mice treated with a novel bifunctional compound, 11β-dichloro. A ligand for the androgen receptor linked to a reactive *N,N*-bis-(2-chloroethyl)aniline mustard composes the 11β-dichloro molecule. These chemical features enable 11β-dichloro to form covalent DNA adducts capable of binding the androgen receptor. It is proposed that androgen receptor-DNA adduct complexes interfere with both DNA repair and androgen receptor function (see Fig. 1). We have shown that 11β-dichloro rapidly induces apoptosis in LNCaP prostate cancer cells and that the new compound can prevent tumor growth *in vivo* (12). This earlier study found that it is essential to have the DNA-damaging and androgen receptor ligand moieties in the same molecule to observe potent activity against cancer cells.

The results of this study show that, following administration by i.p. injection, the intact 11β-dichloro molecule forms DNA adducts in cells in culture as well as in mouse tissues after absorption and distribution. Levels of radioactivity were widely distributed in tissues after administration of [^{14}C]-11β-dichloro. Chromatographic analysis of radiolabeled compounds extracted from blood revealed that intact 11β-dichloro was the predominant molecular species present for up to 4 hours. Several unidentified metabolites represented approximately one third of the radioactivity present in blood at that time.

These findings answer a key question about the relevance of our proposed mechanisms of action of 11β-dichloro to the antitumor effects of this compound *in vivo*. The fact that the compound remains intact and capable of forming DNA adducts in cells and tissues is consistent with the view that its combination of biochemical functions may underlie its ability to prevent tumor growth.

The 11β-guanine adduct we identified is consistent with the major adduct formed by reaction of bifunctional aniline mustard drugs with DNA (15–18) and is the product of the reaction of one arm of the *N,N*-bis-(2-chloroethyl) group with a guanine base. Reaction of the second arm of this group with another DNA base can produce intrastrand or interstrand cross-links (17–19). Evidence indicates that these bifunctional adducts are primarily responsible for the lethal effects of this class of compounds (20, 21). The efficiency of cross-link formation by nitrogen mustards is not great. Thus, any change in the balance between the removal of 11β-monoadducts from DNA and their conversion to cross-links will likely have a significant effect on toxicity. Whether the ability of 11β-DNA adducts to bind the androgen receptor affects this balance requires further investigation.

We found that the levels of 11β-DNA adducts formed in LNCaP xenograft tumors after a single administration of 11β-dichloro were in the range of the adduct levels associated with concentrations that inhibited the growth of LNCaP cells in culture. The ED_{50} for growth inhibition of LNCaP cells in culture by 11β-dichloro is 5.2 μmol/L. Approximately 0.25 adduct per million DNA bases was found in LNCaP cells after a 4-hour exposure to 2.5 μmol/L 11β-dichloro in culture. A single dose of 50 mg/kg 11β-dichloro resulted in a similar level of DNA damage in LNCaP xenografts (average, 0.2 adducts/10^6 bases).

The measured adduct levels in LNCaP cells within xenograft tumors, however, are likely to vary because of the heterogeneity of the tumor microenvironment. Whereas cells in culture experience uniform exposures to 11β-dichloro in media, vascularization and rates of perfusion vary within tumors resulting in nonuniform exposures (22, 23). Thus, the levels of DNA adducts we identified should be considered an average with some cells likely having greater amounts of DNA damage and cytotoxicity from 11β-dichloro whereas others likely experience less.

The levels of 11β-DNA adducts in liver were 8-fold greater than in tumor tissue. This finding led us to investigate whether single or repeated treatments with 11β-dichloro resulted in hepatotoxicity. In our examination of four serum enzymes that are diagnostic for liver disease, we found that significant increases in both alanine aminotransferase and aspartate aminotransferase occurred at the 50 mg/kg 11β-dichloro dose. No increases were found in either γ-glutamyl transferase or alkaline phosphatase. This pattern is consistent with drug-induced injury to hepatocytes after the single dose. At a lower repeated dose of 30 mg/kg over a 7-week period with five consecutive daily doses, none of the four serum enzymes levels were elevated. The fact that inhibition of tumor growth occurred when animals were treated repeatedly with the 30 mg/kg dose in the absence of significant toxicity is encouraging (12).

We propose that in addition to inhibiting DNA repair, association of the androgen receptor with DNA adducts may antagonize androgen receptor transcriptional activity. This unique mechanism of disabling androgen receptor–mediated gene transcription in hormone-refractory prostate

cancer may prove effective against cancers in which overexpression or mutation of the androgen receptor or the changes in androgen receptor coregulators underlie escape from androgen blockade (24–26).

Targeting androgen receptor function in hormone-refractory prostate cancer is often ineffective because of the variety of mechanisms that enable tumor cells to defeat current antihormonal therapies. Nonetheless, the androgen receptor remains an attractive therapeutic target for this disease because of its continued role in the growth and survival of advanced prostate cancers. Elimination of androgen receptor function can leave cells vulnerable to other chemotherapeutic agents such as those that act by damaging DNA (7). We propose that 11β-DNA adducts that capture the androgen receptor could provide a novel way of antagonizing its biological functions in hormone-refractory prostate cancer. The combined effects of persistent DNA damage and the unique mechanism of receptor antagonism by 11β-DNA adducts may result in the disruption of biochemical pathways and provoke prostate cancer cells into apoptosis, leading to more effective therapy.

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